

(51) International Patent Classification ⁴ : G01N 21/64		A1	(11) International Publication Number: WO 87/ 04247 (43) International Publication Date: 16 July 1987 (16.07.87)
(21) International Application Number: PCT/US87/00088 (22) International Filing Date: 14 January 1987 (14.01.87) (31) Priority Application Number: 818,721 (32) Priority Date: 14 January 1986 (14.01.86) (33) Priority Country: US (60) Parent Application or Grant (63) Related by Continuation US 818,721 (CIP) Filed on 14 January 1986 (14.01.86) (71)(72) Applicant and Inventor: LEVIN, Herman, W. [US/ US]; 1919 Chestnut Street, Suite 2706, Philadelphia, PA 19103 (US).			(74) Agent: ELMAN, Gerry, J.; Elman Associates, Bourse Building, Suite 900, Philadelphia, PA 19106-2523 (US). (81) Designated States: AT (European patent), BE (Euro- pean patent), CH (European patent), DE (European patent), FR (European patent), GB (European pa- tent), IT (European patent), JP, LU (European pa- tent), NL (European patent), SE (European patent), US. Published With international search report.

It is proposed to increase the utility of intracellular fluorescence and absorbance measurements for control of fermentation and cell culture by correcting on-line for background fluorescence of the media, also to be able to measure the fluorescence or absorbance of the fluid media in the presence of suspended cells or particles. The measurements could be extended to measure the optical properties of other fluids which contain suspended particles. This invention uses the characteristics of the evanescent wave at the surface of an optic waveguide. The wave penetrates into the less dense medium only to about the depth of about $1/2$ wavelength. The thickness of media swept by the evanescent wave is much less than the size of a cell, thereby essentially separating the fluorescence or absorbance of the media from that of the cells. Apparatus is disclosed for carrying out the methods taught herein, including the use of an optical fiber to generate the evanescent wave and the use of a flat plate waveguide (1316) to generate it. Apparatus that can read both bulk fluorescence and evanescent wave fluorescence employs a flat plate waveguide (1316) to generate the evanescent wave and fiber optics (1305), (1317), (1319) and (1320) to create an alternating dual-beam approach to generating both sets of data.

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AT	Austria	FR	France	ML	Mali
AU	Australia	GA	Gabon	MR	Mauritania
BB	Barbados	GB	United Kingdom	MW	Malawi
BE	Belgium	HU	Hungary	NL	Netherlands
BG	Bulgaria	IT	Italy	NO	Norway
BJ	Benin	JP	Japan	RO	Romania
BR	Brazil	KP	Democratic People's Republic of Korea	SD	Sudan
CF	Central African Republic	KR	Republic of Korea	SE	Sweden
CG	Congo	LI	Liechtenstein	SN	Senegal
CH	Switzerland	LK	Sri Lanka	SU	Soviet Union
CM	Cameroon	LU	Luxembourg	TD	Chad
DE	Germany, Federal Republic of	MC	Monaco	TG	Togo
DK	Denmark	MG	Madagascar	US	United States of America
FI	Finland				

- 1 -

EVANESCENT WAVE BACKGROUND-FLUORESCENCE/ABSORBANCE DETECTION

Background of the Invention5 Field of the Invention

The present invention relates to method and apparatus for the detection and measurement of certain optical characteristics of biological culture and fermentation media in a bioreactor or the like, and more particularly, to the measurement of the fluorescence and/or
10 absorbance of a continuous phase in the presence of a discontinuous phase.

Description of Related Art

Fluorescence of intracellular NADH or NADPH has been shown to be a
15 good indicator of the metabolic state of cells in culture, as well as serving as an indicator of the concentration of cells in the culture medium. Several papers have attested to the value of this kind of in situ measurement for both microbial and yeast culture applications. See, e.g., W.B. Armiger et al., Analysis and Control of Fed-Batch
20 Fermentations Producing Escherichia coli Using Culture Fluorescence, Proceedings Biotech 84, Washington, D.C. 1984. Apparatus for performing these measurements is available from BioChem Technology, Inc., Malvern, Pennsylvania, as the FluoroMeasureTM System.

From an economic point of view, it would be advantageous to use
25 lower cost complex nutrients, such as molasses or corn steep liquor in industrially significant cultures. These, however, introduce additional background fluorescence. If the medium contains a fluorescent component which does not change during the course of the culture, a background correction can be made simply by subtracting the reading at time zero
30 from all subsequent readings. In a case where the fluorescence of the media changes due to use by the cell or in the case where the cells produce a competing fluorescence, it becomes more difficult to correct on-line for changes in background. This can be done for a batch culture by taking serial samples, removing the cells and measuring the
35 fluorescence of the medium. Even more difficult are background corrections in cases of continuous culture or where nutrients are added stepwise during the culture.

For fluorescent media, it would be useful to be able to determine how the fluorescence of the media is changing during the fermentation, such that a measurement can be made as to the metabolic state of the cells and their growth rate. What is needed is a means of effecting the separation of the cell fluorescence from that of soluble materials.

Optical sensors for fermentations or tissue culture are capable of giving information on intracellular substances and conditions. Such information would permit a finer control based on actual intracellular information rather than on the existing on-line sensors, temperature, pH, dissolved oxygen, off-gas analysis. It would lead to a better scaleup and commercialization of products derived from recombinant DNA and cell fusion technologies. Optical sensors, at present, work best with media which do not interfere since there is no easy way to correct for changes in optical background.

The early work with optical sensors for following intracellular metabolism dates back to 1957, when Duysen and Ames observed that the fluorescence of baker's yeast was similar to that of NADH and that the fluorescence of starved yeast could be enhanced by adding ethanol or glucose to the suspension. Later, Harrison and Chance built an instrument capable of measuring culture fluorescence in situ and could monitor aerobic/anaerobic transitions in continuous culture. Using a similar device, Humphrey and coworkers, and others, have shown that a fluorometer placed on a fermentor could measure intracellular NADH changes and might be useful for process control. Zabriskie and Humphrey showed the linear relationship between the logarithm of the fluorescence of the culture and the logarithm of cell concentration. Ristroph et al. studied the relationship between culture fluorescence and the growth of Candida utilis in a fed batch fermentation.

These studies have shown that the concentration in intracellular NADH measured by culture fluorescence in a fermentation is a function of the number of cells, the energy level within each cell, and the level of metabolic activity. A mathematical expression which is derived from these studies is:

$$F(t) = [Y_{f/x}(1+m(t))]X(t) + E(t)$$

X(t) is the cell concentration. The term in square brackets is the

- 3 -

fluorescence yield, which is made up of an invariant component $Y_{f/x}$, which is characteristic of the type of organism and a variable component $m(t)$, which changes in response to shifts in the level of metabolic activity. The final term, $E(t)$, with which the present invention is
5 mainly concerned, is the environmental, or background, fluorescence. Obviously, if $E(t)$ fluctuates during the fermentation, then it would be difficult if not impossible to derive information about the cells from the measured overall fluorescence. Continuous, or batch fed
10 fermentation or cell cultures only exacerbate the problem. In those techniques, additional variables are introduced without corresponding information as to concentration.

Almost all of the published studies have used synthetic media where $E(t)$ is low, or the corrections for $E(t)$ had to be arrived at empirically. In scaling up fermentations and cell cultures for
15 commercial production, economic factors may dictate use of the natural nutrients, like molasses or fetal calf serum, which have a natural fluorescence and therefore contribute to the background value. When checking some of the assumptions used in correcting for the background, I found indications that the background fluorescence of, for instance,
20 molasses, and the fluorescence of yeast cells do not add linearly. This pointed up the need for a method for continuously measuring the media fluorescence background on-line and in real time, i.e. using a sensor or sensors continuously monitoring the detected variable as the fermentation or culture is being conducted.

25 This means that, without physically separating the cells from the media, a method was needed which caused the media to fluoresce without, at the same time, causing the cells to fluoresce. In accordance with the present invention, the evanescent wave phenomenon is used to meet this need.

30

35

Summary of the Invention

When a beam of light is totally reflected from a non-mirrored interface between two optically transparent media of different refractive indexes, an evanescent wave phenomenon, such as shown in Fig.

- 5 1, exists. The light beam 11 is totally reflected from this kind of surface, unlike a mirrored surface, and behaves as though it penetrates for about half a wavelength into the less dense medium 12, e.g. an aqueous medium. Reference numeral 15 identifies the portion of light beam 11 that is the evanescent wave in the less dense medium 15.
- 10 Reference numeral 16 identifies a measuring arrow showing a distance that is one wavelength of the light beam 11.

Figs. 1 and 2 schematically show that the reflected beam is slightly displaced from where it would be if reflected from a mirrored surface. This displacement has been shown experimentally, and it is one of the

15 proofs of the existence of the evanescent wave. This part of the light beam has many characteristics of a standing wave parallel to the surface. Fig. 3 shows how the intensity decreases with distance from the surface.

In Fig. 3, N is the incident wave, R is the reflected wave Θ is the

20 angle of incidence (which is greater than Θ_c , the critical angle. Z is the distance axis in the rarer medium measured from the interface with the more dense medium. E_0 is the initial magnitude of the electric field component of the light at zero depth in the rarer medium. dp is the depth of penetration, defined as the distance required for the

25 electric field to fall to e^{-1} of its value at the surface. The value of dp is directly related to the wave length in the denser medium and is inversely proportional to the angle of incidence and top the ratio of refractive indexes of the two media. The greatest strength of the evanescent wave occurs at the surface, and it decreases exponentially

30 with distance from the surface.

It can be absorbed by an appropriate colored material, and if the material is fluorescent, it can excite the material to fluoresce. At the wave length of interest, 340 nm, the volume in liters swept out by this evanescent wave over a one square centimeter area would be 1.7×10^{-8} liter. For a 200 um diameter optical fiber 2.5 cm long, the swept

35 volume would be 1.3×10^{-11} liter.

The present invention accomplishes this separation by using the

- 5 -

characteristics of the evanescent wave which forms in the less dense medium when light is totally reflected from the interface between two optically transparent substances of different refractive indexes. It makes use of my observation that it is unlikely that an intact cell will
5 be in the volume of fluid swept by the evanescent wave next to the optical waveguide since the wave penetrates approximately only $1/3$ to $1/2$ of a wavelength into the aqueous layer. The present invention therefore contemplate measurement of the fluorescence of the medium without interference from the intracellular fluorescence or from fluo-
10 rescence of particles in solution.

The same concept is also adapted to measuring the optical absorbance of the medium independent of cells and particulate material. This can significantly reduce the complexity of the computer programs needed to deconvolute the data and thereby make the control of
15 fermentation and tissue culture easier to achieve.

At the usual concentration of cells in a bioreactor, it is unlikely, as I said, that a cell would be in this small volume of fluid at any given time. Also, since, at an excitation wavelength of for example 280 nm, the evanescent wave only penetrates about 110 to 170 nm
20 into the liquid phase, even if the cell is resting right on the surface of the optical wave guide, very little of the cell volume (mostly the cell wall or cell membrane), will interact with the evanescent wave. Thus, by limiting the volume that can interact with the light wave to that within the evanescent wave, the present invention provides, in
25 effect, a separation of the intracellular fluorescence or absorbance from the fluorescence or absorbance of the media.

It is an object of this invention to facilitate the opening up of fermentation and cell culture to intracellular optical measurement under a wider variety of culture conditions because there will be a way to
30 correct continuously for environmental or background changes on-line in real time.

It is a further object of the present invention to provide for the separation of the optical effects of intracellular contents from the optical effects of the culture medium.

35 It is a further object of the present invention to follow changes in the culture medium without interference from changes in the intracellular contents.

It is a further object of the present invention to follow changes in the intracellular contents without interference from changes in the culture medium.

It is a still further object of the present invention to reduce
5 costs of monitoring the fluorescence of cell cultures.

It is a still further object of the present invention to simplify and shorten the time for scaleup or change of media in a fermentation, since separate, offline empirical measurements of background or environmental fluorescence will not have to be made. By a single test
10 run, culture conditions might be brought to a preliminary optimization by appropriate additions of media components.

It is a still further object of the present invention to provide information about the extent of cell rupture, whether due to shear forces or other mechanical or chemical causes. In accordance with the
15 present invention, the effect of stirring forces on cell integrity could be measured on-line in real time, allowing corrections to be made during a fermentation run rather than after the run when data shall be subsequently analyzed.

It is a still further object of the present invention to permit the
20 use of optical absorbance methods for following other non-fluorescent intracellular materials, since the ability to correct for background would provide the equivalent of a continuous dual beam spectrophotometer.

It is a still further object of the present invention to provide for
25 the better control of the concentration of individual nutrients in the culture media through the improved ability to follow specific changes of optically differentiable materials in the medium.

It is a still further object of the present invention to significantly improve the yield of fermentation and tissue cultures,
30 reduce the time and cost of scaleup, allow for a more precise control based on the state of the intracellular metabolism, and speed up the commercialization of new recombinant DNA and cell fusion technologies through more efficient fermentation and tissue culture techniques.

35 Brief Description of the Drawings

Fig. 1 is a schematic illustration of the evanescent wave phenomenon, wherein a light beam 11 traverses a path from left to right

through a wave guide 13 such as an optical fiber.

Fig. 2 is an enlarged view of the circular area 2 within Fig. 1.

Fig. 3 is a schematic illustration of the variation of intensity of the evanescent wave with distance from the interface between the two
5 media.

Fig. 4 is a partially schematic illustration of a cross-sectional elevation of one of the variations of the present invention employing a metal sheathed optical fiber that may be dipped into a liquid medium.

Fig. 5 is a cross sectional side elevation of the embodiment
10 shown in Fig. 4 viewed from a right angle to the view of Fig. 4, taken along the cross sectional line 5--5.

Fig. 6 is a partially schematic illustration of another embodiment of the present invention.

Fig. 7 shows the portion of the embodiment of Fig. 6 viewed from a
15 right angle to the view of Fig. 6, taken along the line 7--7.

Fig. 8 is a partially schematic illustration of a cross-sectional elevation of a preferred embodiment of the present invention wherein the optical waveguide is a flat plate 1316, and a barrier 1312 confines the liquid to one side of the waveguide.

Fig. 9 is an elevation view of the disk 1328 shown in Fig. 8
20 taken along the line 9.

Fig. 10 is a cross sectional side elevation of the embodiment shown in Fig. 8 viewed from a right angle to the view of Fig. 8, taken along the cross sectional line 10--10.

Fig. 11 is an enlarged view of the area 11 within Fig. 8 .
25

Fig. 12 is a schematic illustration of the embodiment of Fig. 8 showing the paths of light rays and the steps of processing of data.

Fig. 13 is a schematic illustration of the contents of the source and detector housing of an embodiment alternative to the design shown
30 housed in element 1331 of Fig. 8.

Fig. 14 shows a portion of the embodiment of Fig. 13 viewed from a right angle to the view of Fig. 13 taken along the line 14.

Detailed Description

The embodiment of the invention shown in Figs. 4 and 5 provides a relatively simple means of determining the background fluorescence of a solution containing particulate matter by dipping into the liquid a dipstick 40 which comprises a metal housing 41 surrounding an optic fiber 44 through which an evanescent wave of light is used to excite the fluorescence of the solution. The housing 41 defines a chamber 47, into which the solution to be tested passes through apertures 46 in the housing 41.

On the portion of the optic fiber 44 within the chamber 47, the opaque sheath 42 and transparent cladding 43 have been removed, exposing the fiber 44 directly to the solution within the chamber 47.

To illuminate the fiber 44, there is provided a light source 1301 of either visible or invisible light, such as an incandescent lamp or laser, an excitation beam lens 1302, and an excitation beam filter 1303, arranged as shown in Fig. 4.

The radiation from the light source 1301 passes through the lens 1302, where it is collimated, and then passes through the filter 1303, where it is filtered into a monochromatic excitation beam 1326 of desired wavelength (schematically illustrated by dashed lines with arrows) and passes through an aperture in plate 1340.

The excitation beam 1326 thereupon encounters a dichroic mirror 1349 adapted to reflect the wavelength of light represented by the excitation beam 1326 into the excitation-emission beam lens 1358, where the beam 1326 is decollimated and directed into the proximal end of optic fiber 44, which may extend from the metal housing 41 if desired. As stated above, the excitation beam 1326 travels through optic fiber 44 until it reaches the distal end, where it encounters a light trap 45, e.g. of black silicone rubber.

The evanescent wave portion of the excitation beam 1326 traveling down the optic fiber 44 encounters the molecules of solute within chamber 47 that are immediately adjacent the fiber 44 (i.e. within about 1/3 to 1/2 wavelength as discussed above) and, to the extent that it is susceptible, excites the solute to emit fluorescence.

Such fluorescence passes into the optic fiber 44 and travels to its proximal end as the emission beam 1327, where it exits the optic fiber 44, encounters the excitation-emission beam lens 1358 and is collimated.

The beam 1327 then passes through the dichroic mirror 1349, which has been fashioned to pass the wavelength of the light emitted by the fluorescence of the solute to be measured.

The emission beam then impinges upon an electronic detection system 5 1323 such as a photomultiplier tube in counting mode. The detection system 1323 is selectively responsive to the wavelength of the emission radiation because a filter or monochromator is incorporated therein. The electronic detection system 1323 generates a signal that is sent to a lock-in ratio amplifier 1344. A reference detector 1350, which 10 detects the intensity of the excitation beam 1326, also generates a signal that is sent to the lock-in ratio amplifier 1344, where the two signals are processed conventionally to compensate for variations in the intensity of the excitation beam 1326.

The signal from the lock-in ratio amplifier 1344 is transmitted to 15 an analog-to-digital convertor 1345, which generates a signal fed to a digital display panel or signal processor 1346. Desirably an additional digital signal 1352 from a conventional device reading bulk fluorescence (of the solution and any particulate matter in it) is generated and similarly fed to the display panel or processor 1346. The signal 1352 20 may, for example, be from a FluoromeasureTM fluorometer (BioChem Technologies, Inc., Malvern, Pa.). When the data in signal 1345 is subtracted from the data in signal 1352, the resulting data sent to element 1351 describes the fluorescence of the particulate matter, inas- 25 much as fluctuations in the fluorescence of the solute have been subtracted from the fluorescence of the bulk.

An alternative embodiment shown in Figs. 6 and 7 utilizes an optic fiber 62 within a housing 61 defining a chamber 67 having a pair of 30 ports 72, 73. The slurry to be subjected to optical measurement in accordance with the present invention may be introduced through inlet port 72 and exhausted through outlet port 73. Desirably the chamber 67 is designed so that flow therethrough is essentially laminar.

As in the embodiment previously described (Figs. 4 and 5), light 35 from a light source 1301 passing through a lens 1302 and filter 1303 is introduced into the end of the fiber optic 62. However, to maximize the evanescent wave relative to the radiation traveling straight through the fiber optic 62, a plate 1340A, having an O-shaped aperture, with a

filled-in center, may be used rather than one having a circular cutout. This will block rays from entering the fiber optic 62 along the axis.

In this configuration, a reference detector 1350 is disposed along a different path from the light source 1301 than that traveled by the
50 excitation beam 1326 so that variations in the intensity of the light source can be detected and fed to a lock-in ratio amplifier 1344 as is conventional.

At the distal end of of the fiber optic 62, an electronic detection system 1323 is placed to receive light traveling therethrough. The
100 detection system 1323 may be set up to detect the intensity of light of the wavelength of the emission beam 1326. In that event, it will generate a signal useful in determining the absorbance of the solute in solution, free of cellular or other particulate matter. The absorbance information may be related to the concentration of a solute that is to
15 be monitored, or it may be a background figure which may appropriately be subtracted from another absorbance reading to provide useful data.

Alternatively the detection system 1323 may be set up with an appropriate filter or monochromator to detect a wavelength of radiation which is emitted by a solute as fluorescence, in which event the resul-
20 ting data will be similar to that generated by the embodiment of the invention shown in Figs. 4 and 5.

Similarly to the previously described embodiment, the signal from detection system 1323 is supplied to the lock-in ratio amplifier 1344, and the output thereof is directed to a display panel or processor 1346,
25 the output of which may, for example, be fed to a chart recorder 1353 as shown.

Using the evanescent wave phenomenon, the embodiment of the present invention shown in Figs. 8 to 11 is capable of determining both
30 absorbance and fluorescence in such quick alternating succession as to provide virtually simultaneous readings. With a flat plate 1316 as the wave guide, the device housed in detector enclosure 1332 is particularly adapted to be used in a reactor or fermentation vessel 1312 for real-time determination of several variables which assist in determining the
35 instantaneous concentration of various components of the contents of the vessel 1312.

The detector enclosure 1332 is mounted within a conventional pipe-

SUBSTITUTE SHEET

- 11 -

like mounting port 1362 extending from the reactor vessel wall 1312. The distal end of mounting port 1362 is threaded to mate with grommet 1333, which secures the enclosure 1332 to the port 1362 and thereby to the reactor vessel wall 1312.

5 Depending on the length of the mounting port 1362 and the depth to which it is desired that the detector housing 1332 penetrate beyond the vessel wall 1312 into the reaction mixture 1314, a rubber-like O-ring 1311 is interposed within any of three O-ring grooves 1361 to seal the retainer sleeve 1354 of housing 1332 watertight within the port 1362.

10 A screw-threaded wave guide plate mounting sleeve 1336 mates with the retainer sleeve 1354 and holds the wave guide plate 1316 securely in place. An insert 1335, which may be one of optionally several lengths, extends the wave guide plate mounting sleeve 1336 so that the wave guide plate 1316 extends the desired distance into the reaction mixture 1314
15 beyond vessel wall 1312.

 A fluorescence enclosure 1308 extends outwardly from the vessel wall 1312, screw-threaded to the insert 1335. Enclosed within the aforesaid elements are optical fiber elements 1305, 1317, 1319 and 1320, which convey light to and from the wave guide plate 1316. The optical
20 fiber elements 1305, 1317, 1319 and 1320 pass through fluorescence enclosure cover 1307, which is held in place by a fluorescence enclosure cover retainer bezel 1309.

 The light source 1301, lens 1302, filter 1303 and aperture 1340 are generally as have been described above. As shown schematically in Fig.
25 12 as well as generally in Fig. 8, an excitation beam light chopper 1325 is interposed in the optical path to pass the focused excitation beam 1326 to the evanescent wave excitation fiber optic 1305 and then to the direct wave excitation fiber optic 1320 in alternating succession. As shown more particularly in Fig. 9 the excitation beam light chopper
30 1325 comprises a disk 1328 having mounted thereon a semicircular mirror 1329, the other half of the disk 1328 having an opening 1342 sufficiently wide to allow the excitation beam 1326 to pass through to the optical fiber 1320.

 The chopper disk 1328 is rotated by a shaft 1330. As the disk 1328
35 rotates, the excitation beam 1326 directed into two alternate paths. When the excitation beam 1326 is incident on the mirror 1329, the beam follows path 1326A directed to optic fiber 1305. Alternately, when the

disk 1328 has rotated to a position where the excitation beam 1326 is incident on the opening 1342, it passes through to the optical fiber 1320.

5 The optical fiber 1305 carries the excitation beam 1326A from the sealed, light-tight housing 1331, to fluorescence detector enclosure 1332. The optical fiber 1305 typically consists of several individual fibers completely surrounded by a flexible transparent cladding 1306 and an opaque flexible sheath 1304. The refractive index of the transparent cladding 1306 is slightly less than that of the optic fiber
10.5 1305.

After passing through grommet 1333, the individual optical fibers of the optic fiber 1305 pass through a mounting block 1355 where they are spread out, as shown in Fig. 10. Optic fiber mounting block 1355 is desirably injection molded of a plastic capable of withstanding
15.5 sterilization temperature of about 140 °C, e.g. polysulfone.

The individual fibers of optical fiber 1305 that are in contact with the prism 1310 along its oblique surface are cut square to the longitudinal axis of the fibers. The mounting block 1355 holds the fiber bundle 1305 such that the light enters at right angles to the
20 oblique surface of the prism 1310. The angle of the oblique surface of prism 1310 to the side of the prism in contact with the flat plate wave guide 1316 is such as to introduce the light beam 1326A into the flat plate wave guide 1316 at an angle greater than the critical angle, so that the light beam 1326A will be confined to the flat plate wave guide
25 and will generate an evanescent wave at the interface of the wave guide 1316 and the reaction medium 1314 in contact with the wave guide.

Prism 1310 is rectangular and has the same refractive index as the flat plate wave guide 1316. The loss of intensity of excitation beam 1326 during its transition from the optic fiber 1305 to the prism 1310
30.5 is minimized by having the sides of the prism 1310 greater than the diameter of the optical fiber 1305 and by having the transitional interface between the optic fiber 1305 and the slanted surface of the prism 1310 covered by a liquid 1324 having same refractive index as that of the prism 1310.

35 The flat transparent side of the prism 1310 is cemented to the distal face 1337 of the flat plate wave guide 1316 by utilizing a transparent cement having a same refractive index as that of prism 1310

SUBSTITUTE SHEET

and flat plate wave guide 1316. The actual angle of the oblique surface of prism 1310 is a function of the refractive indices of the materials used for prism 1310 and the flat plate wave guide 1316. The flat plate wave guide 1316 is circular in cross section and is typically made of

5 bubble-free and distortion-free material such as quartz. The two parallel faces 1337 and 1338 are optically polished to a high degree and are truly parallel within the the normal manufacturing tolerances. The cylindrical side wall of the flat plate wave guide 1316 is significantly less in height than its diameter.

10 The flat plate wave guide 1316 is sealed along its side wall by an opaque seal 1356 of PTFE polymer (e.g. Teflon or the like) to prevent loss of light and also act as a sealant. The frontal side 1338 of the flat plate wave guide 1316 is coated with a very thin and hard

15 transparent layer 1315 of material such as Surlyn (Dupont), deposited diamond etc. The thickness of the wave guide coating 1315 should be such as to have no effect on the penetration of the evanescent wave 1339 into the medium 1314. The wave guide coating 1315 is desirable to prevent the adherence of cellular products generated by the

20 wave guide coating 1315 also prevents damage such as scratches to the frontal side 1338 of the flat plate wave guide 1316.

An evanescent wave 1339 is created within the flat plate wave guide 1316 when the excitation beam 1326 is repeatedly reflected between the two non-mirrored surfaces 1337 and 1338 respectively. The evanescent

25 wave 1339 so generated penetrates into the medium 1314 under observation through the frontal side 1338 of the flat plate wave guide 1316. As explained above, the evanescent wave only penetrates a distance up to about half of a wave length of the excitation beam 1326 into the medium 1314 under observation. The discrete particulate matter 1313 such as

30 cells present in the medium 1314 has virtually no interaction with the evanescent wave 1339.

There is a prism arrangement 1341 similar to prism 1310 at the opposite end of the flat plate wave guide 1316 along its distal side 1337 as shown in Figs. 8 and 12. The thickness of the flat plate wave

35 guide and the distance between the prisms 1310, 1341 is such that the incident light beam 1326A is refracted an integral number of times and exits through prism 1341. The ends of the individual fibers of fiber

optic 1317 have been cut square to the longitudinal axis of the fibers. The mounting block 1355 holds the fiber bundle 1317 such that its longitudinal axis is at right angles to the oblique surface of prism 1341.

5 As an alternate construction, the flat plate wave guide 1316 and prisms 1310, 1341 may be fabricated as a single unit. Moreover, alternatively to the relationship illustrated herein, wherein the faces of the prisms 1310, 1341 are raised above the surface of the wave guide 1316, the oblique faces of the prisms may be recessed into the surface
10 of the wave guide. As an additional alternative, two diametrically opposite edges of the wave guide plate may be beveled to serve an equivalent function to the oblique edges of prisms 1310 and 1340.

Individual fibers of the optical fiber 1317 are attached to an oblique surface of the prism 1341, the transitional interface consisting
15 of a liquid film 1324 having a refractive index close to that of the flat plate wave guide 1316. The optical fiber 1317 then passes through the optic fiber mounting block 1355 and its cross section then becoming circular. The optical fiber 1317 then passes through the grommet 1333 and enters the source and detection housing 1331. In direct path of
20 optical fiber 1317 as shown in Fig. 8 there exists a light chopper assembly 1318 which is constructed similarly to excitation light chopper 1325. Next to the light chopper assembly 1318 in the same direction there is an emission beam filter 1321 which eliminates all the nonfluorescent light, followed by the emission beam lens 1322 which
25 focusses the emission beam 1327 on an electronic detection system 1323.

Fig. 12 is a schematic representation of the light path in the embodiment of the present invention shown in Fig. 8. The light beam 1326 from light source 1301 is focused by a lens 1302 such that the
30 light is properly coupled to the optical fiber bundles 1305 and 1320. In coupling light to the optical fibers or wave guides, attention must be paid to the numerical aperture ("NA") of the fiber optic or wave guide. It is a matter of matching the lens 1302 to the NA and the diameter of the fiber or fiber bundle or quartz wave guide.

35 Care must be taken to prevent the fiber cladding from acting as a wave guide. This can be achieved by sheathing the cladding with an opaque sheath. To get uniform distribution of light, the lens must

SUBSTITUTE SHEET

- 15 -

confine the light uniformly across the input face of the fiber or wave guide. The light then passes through a slit or diaphragm and thence to chopper 1325.

Chopper 1325 then, by alternately interposing and removing the
5 mirror 1329 from the excitation light beam 1326, divides the light beam 1326 into two paths, 1326A and 1326B.

Beam 1326A travels through fiber bundle 1305 to prism 1310, which couples the light beam properly to flat plate wave guide 1316 such that the light beam is guided by multiple internal reflection through the
10 wave guide. The evanescent wave is absorbed by the solution components able to interact with light of the selected wavelength. The evanescent wave does not optically interact with or excite to fluorescence the particles 1313 suspended in the reaction medium 1314. Those solution components able to fluoresce will emit their fluorescence at or near the
15 surface of the flat plate wave guide 1316.

A portion of the emitted light will couple into the wave guide and be transmitted through light path 1327A to chopper 1318. Light beam 1326B is transmitted via fiber bundle 1320 to the internal surface 1337 of the flat plate wave guide, which acts as a window; since the light
20 impinges at right angle to the surface, it goes right through and illuminates the bulk suspension near the flat plate 1316, thus exciting to fluorescence both the solution 1314 and the particles or cells 1313 suspended therein which are able to fluoresce.

A portion of the emitted fluorescence beam 1327B passes back
25 through the flat plate 1316 and enters fiber bundle 1319 and is guided to chopper 1318. Chopper 1318 is synchronized with chopper 1325 through synchronizer 1348 such that when chopper 1325 is diverting the light beam over pathway 1326A, chopper 1318 is configured to allow light through pathway 1327A to go through filter 1321 and lens 1322 to
30 detector 1323. When chopper 1325 is diverting the light beam over pathway 1326B, chopper 1318 is configured to allow light through pathway 1327B to go through filter 1321 and lens 1322 to detector 1323.

Synchronizer 1348 also serves to synchronize the signal processing train with the chopper positions such that the signal processor is
35 treating the signal as is appropriate to the mode of generation of the signal, e.g. determination of evanescent wave fluorescence vis-a-vis determination of bulk fluorescence.

The signal from the detector 1323 and from synchronizer 1348 is fed, for example, to an AC to DC converter and linearizer 1343, then to a lock-in ratio amplifier 1344, where the amplitude of the signal is corrected for variations in the amplitude of the excitation beam

5 detected by reference detector 1350, then to an analog-to-digital converter 1345, then to a digital display or signal processor 1346 and then to a digital storage or memory 1347.

If filter 1321 is constructed to pass the emitted fluorescence wavelength, then the device of the present invention measures
10 fluorescence. If filter 1321 is constructed to pass the same wavelength as the excitation beam 1326, then the device measures the optical absorbance of the solution 1314.

In the event that it is desired that only fluorescence and not optical absorbance be measured, an alternative embodiment (not shown)
15 may omit the chopper 1318 of Figs. 13 and 16. In that event, light beam 1327A is merely trapped rather than being guided to filter 1321 and detector 1323. Fiber optic 1317, in such an embodiment, may be omitted and replaced with a light trap, or fiber optic 1317 may itself channel the light away from the flat plate wave guide 1316 as a light trap.
20 Both the bulk fluorescence excited by light beam 1326B and the solution fluorescence excited by light beam 1326A generating an evanescent wave at the surface of the flat plate wave guide 1316 are transmitted over light path 1327B to filter 1321. Filter 1321 is selected to allow only the emitted fluorescent wavelength to pass through.

25

Fig. 13 illustrates yet another embodiment where the contents of housing 1360 are substituted for the contents of housing 1331 of the embodiment of Fig. 8. This embodiment is suitable for slow speed chopping of the light beams, for example, a few hertz or even fractions
30 of a hertz. The chopping device viewed in the direction of arrow 14 is shown in Fig 14. A flat plate 182 is affixed to a shaft 181, which is attached to a speed reducer clutch 183. The clutch 183 is attached to the shaft of reversible motor 184. A flat mirror 171 is attached to plate 182.

35 The plate 182 with mirror 171 attached is pivoted to swing between the position shown in Fig. 13 with solid lines or alternately the position shown with dashed lines. A stop 172 limits the travel of mirror

SUBSTITUTE SHEET

171 as the plate 182 abuts it and acts as a rigid point fixing the position of the mirror precisely again and again. Mirror 173 is similarly mounted for reciprocation and synchronized with mirror 171, generally as described with respect to the embodiment of Figs. 8 and 12.

List of Illustrated Elements

- 40 Dipstick
- 10 41 Fluorescence Detector Metal Housing
- 42 Optic Fiber Opaque Sheath
- 43 Optic Fiber Transparent Cladding
- 44 Optic Fiber
- 45 Light Trap
- 15 46 Apertures in Housing
- 47 Chamber in Housing
- 61 Housing
- 62 Optic Fiber
- 64 Cladding
- 20 65 Sheath
- 66 Liquid Medium in Chamber
- 67 Chamber
- 72 Inlet Port
- 73 Outlet Port
- 25 171 Swinging Mirror Frame
- 172 Rubber-cushioned Stops (Restrict travel)
- 173 Mirror
- 181 Electric Motor Shaft
- 182 Counter weight
- 30 183 Friction clutch to prevent over torquing mirror
- 184 Low torque reversible electric motor
- 1301 Light source
- 1302 Excitation Beam Lens
- 1303 Excitation Beam Filter
- 35 1304 Opaque Sheath of a typical optic fiber
- 1305 Excitation Fiber Optic--Evanescent Wave
- 1306 Optic fiber transparent cladding

- 18 -

- 1307 Fluorescence Enclosure Cover
- 1308 Fluorescence Enclosure
- 1309 Fluorescence Enclosure Cover Retainer Bezel
- 1310 Excitation Beam Prism
- 5 1311 O-ring
- 1312 Reactor Vessel Wall
- 1313 Particulate Matter
- 1314 Reaction Medium
- 1315 Evanescent Wave Guide Coating
- 10 1316 Flat Plate Evanescent Wave Guide
- 1317 Emission Fiber Optic—Evanescent Wave
- 1318 Emission Beam Light Chopper
- 1319 Emission Fiber Optic—Direct Wave
- 1320 Excitation Fiber Optic—Direct Wave.
- 15 1321 Emission Beam Filter
- 1322 Emission Beam Lens
- 1323 Electronic Detection System
- 1324 Liquid Separator between prisms and Excitation fiber optic and
Emission Fiber Optic—Evanescent Wave
- 20 1325 Excitation Beam Light Chopper
- 1326 Excitation Beam
- 1327 Emission Beam
- 1328 Disc (Typical Design) for Light Choppers
- 1329 Light Chopper Mirror
- 25 1330 Light Chopper Motor Shaft
- 1331 Source & Detector Housing
- 1332 Fluorescence/Absorbance Detector Enclosure
- 1333 Grommet
- 1334 Fluorescence Detector Retainer Flange
- 30 1335 Insert (which may be of various sizes) for wave guide plate
position adjustment
- 1336 Wave Guide Plate Mounting Sleeve
- 1337 Distal Side of the Wave Guide Plate
- 1338 Frontal Side of the Wave Guide Plate
- 35 1339 Evanescent Wave
- 1340 Plate with Aperture
- 1341 Emission Beam Prism

- 19 -

- 1342 Opening in Light Chopper Disk
- 1343 AC to DC Convertor and Linearizer
- 1344 Lock-In Ratio Amplifier
- 1345 Analog to Digital Convertor
- 5 1346 Digital Display Panel or Signal Processor
- 1347 Digital Memory Storage
- 1348 Chopper Synchronizer
- 1349 Dichroic Mirror
- 1350 Reference Detector
- 10 1351 Fluorescence of cells corrected for medium fluorescence
- 1352 Digitized Signal from Bulk Fluorescence Detection Device
- 1353 Strip Chart Recorder
- 1354 Retainer Sleeve
- 1355 Optic Fiber Mounting Block
- 15 1356 Wave Guide Edge Seal
- 1358 Emission-Excitation Beam Lens
- 1360 Source and Detector Housing
- 1361 O-ring groove
- 1362 Mounting Port

20

25

30

35

Claims

Having thus described my invention, what it is desired to protect by Letters Patent and hereby claim is:

5

1. A method for the detection of the fluorescence or absorbance of a continuous phase of a slurry having a continuous phase in the presence of a discontinuous phase which also fluoresces or absorbs light at the wavelengths of interest, comprising the steps of

10

exciting the continuous phase with an evanescent wave, and

detecting the fluorescence resulting therefrom.

155

2. A method for the determination of the fluorescence or absorbance of the discontinuous phase of a system having a continuous phase which also fluoresces or absorbs light at the wavelengths of interest, comprising the steps of

20

exciting the continuous phase with an evanescent wave, and

detecting the fluorescence or absorbance resulting therefrom,

illuminating the system with a non-evanescent wave, and

255

detecting the fluorescence or absorbance resulting therefrom, and

determining the difference between the two values.

30

3. Apparatus for determining the the fluorescence or absorbance of the discontinuous phase of a system having a continuous phase which also fluoresces or absorbs light at the wavelengths of interest, comprising means for

35

exciting the continuous phase with an evanescent wave, and

for detecting the fluorescence or absorbance resulting therefrom, and

- 21 -

for illuminating the system with a non-evanescent wave, and

for detecting the fluorescence or absorbance resulting therefrom and for
and determining the difference between the two values.

5

4. Apparatus of claim 3 including means for performing each step
on a continuous basis in real time.

10 5. Apparatus of claim 3, wherein means for exciting the continuous
phase with an evanescent wave comprises an optical fiber having a
portion of its cladding removed.

15 6. Apparatus of claim 3, wherein means for exciting the continuous
phase with an evanescent wave comprises an optical plate.

7. The method of claim 2 wherein the continuous phase is a
bioreactor and the discontinuous phase comprises living cells.

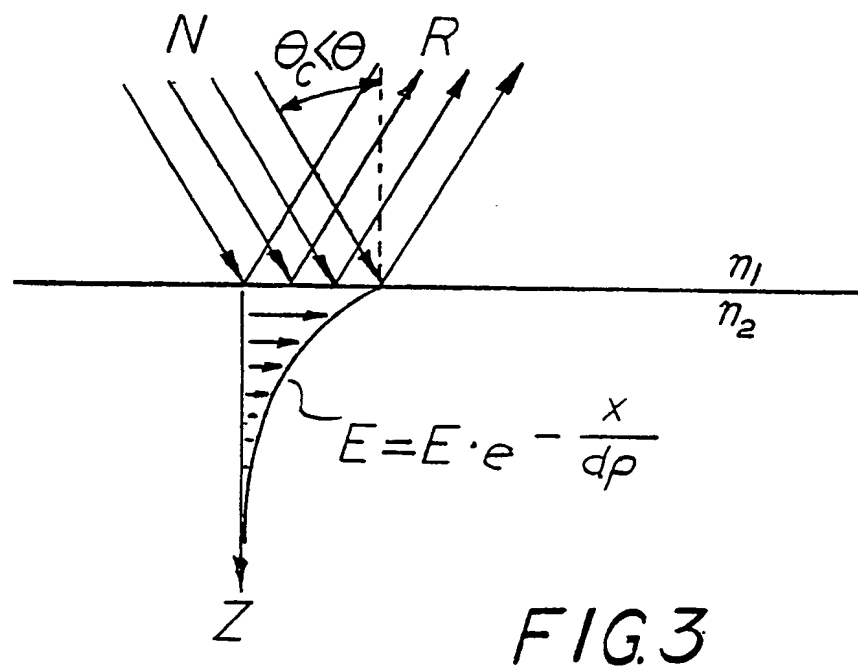
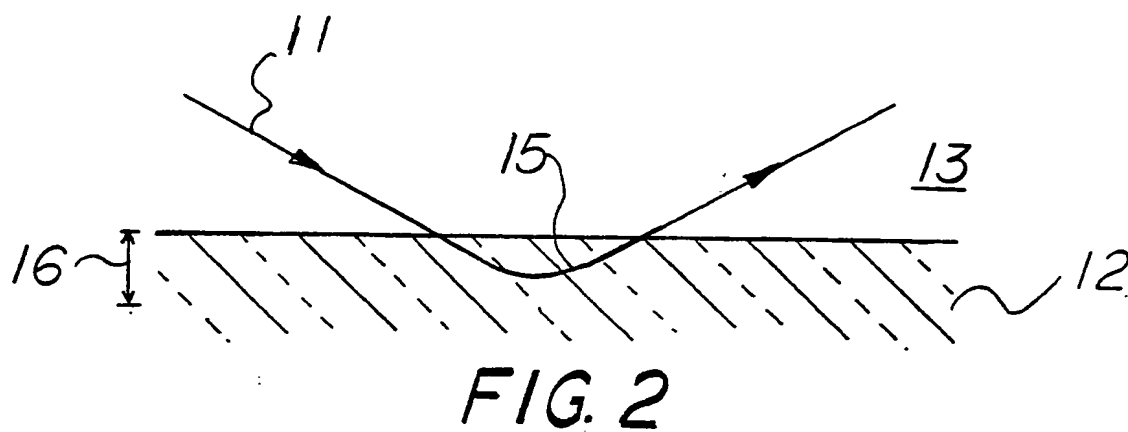
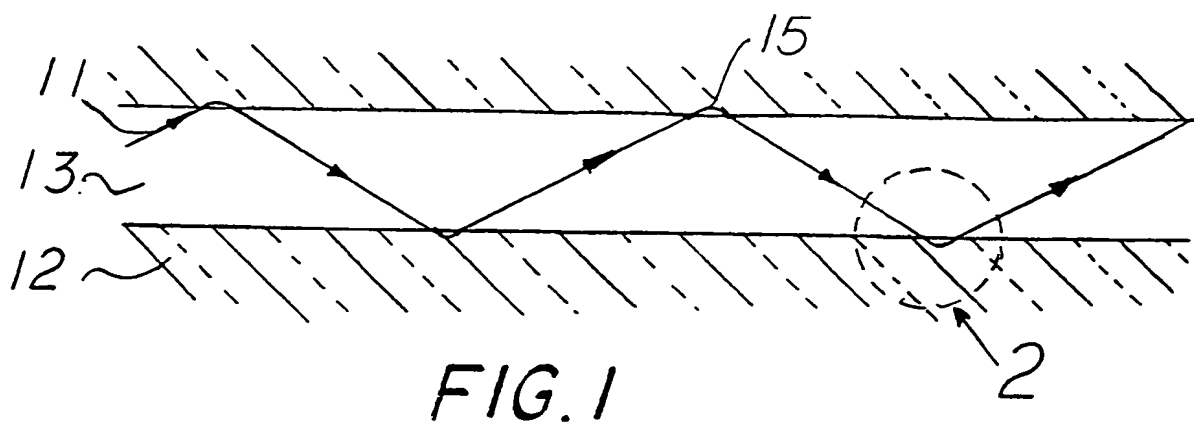
20 8. The method of claim 7 wherein the fluorescence of NADH and
NADPH are measured.

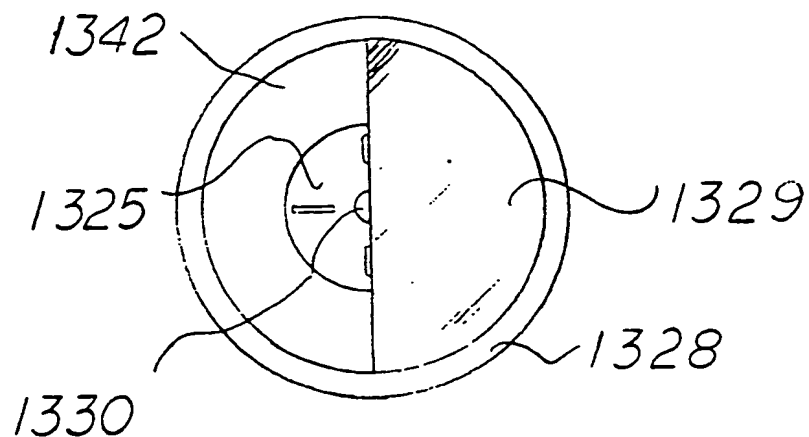
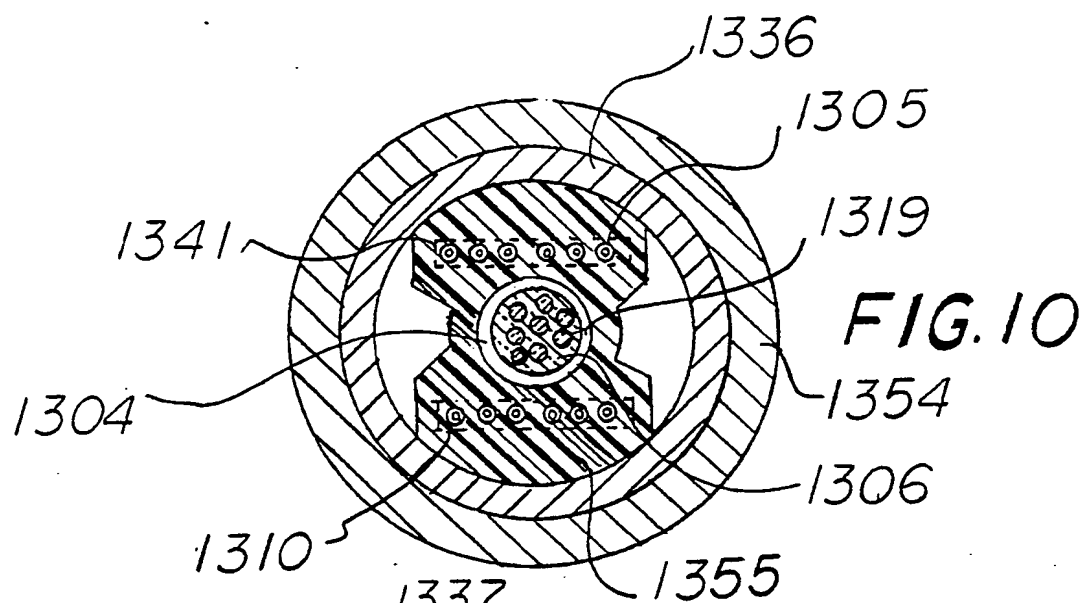
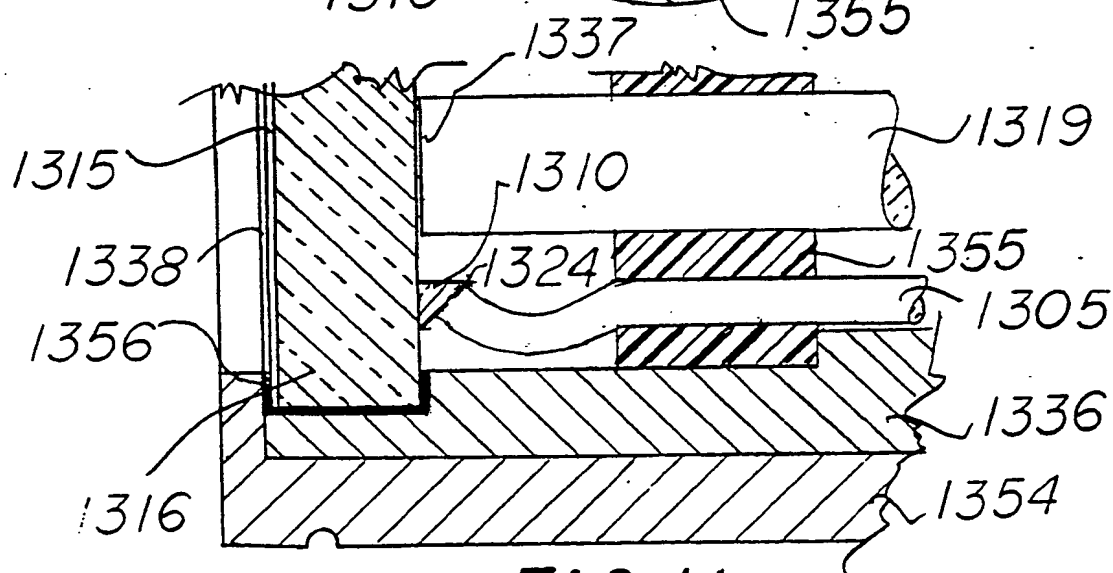
25 9. The method of claim 8 wherein the excitation beam is about 366
nm and the fluorescence is measured at about 460 nm.

30

35

35



**FIG. 9****FIG. 10****FIG. 11**

SUBSTITUTE SHEET

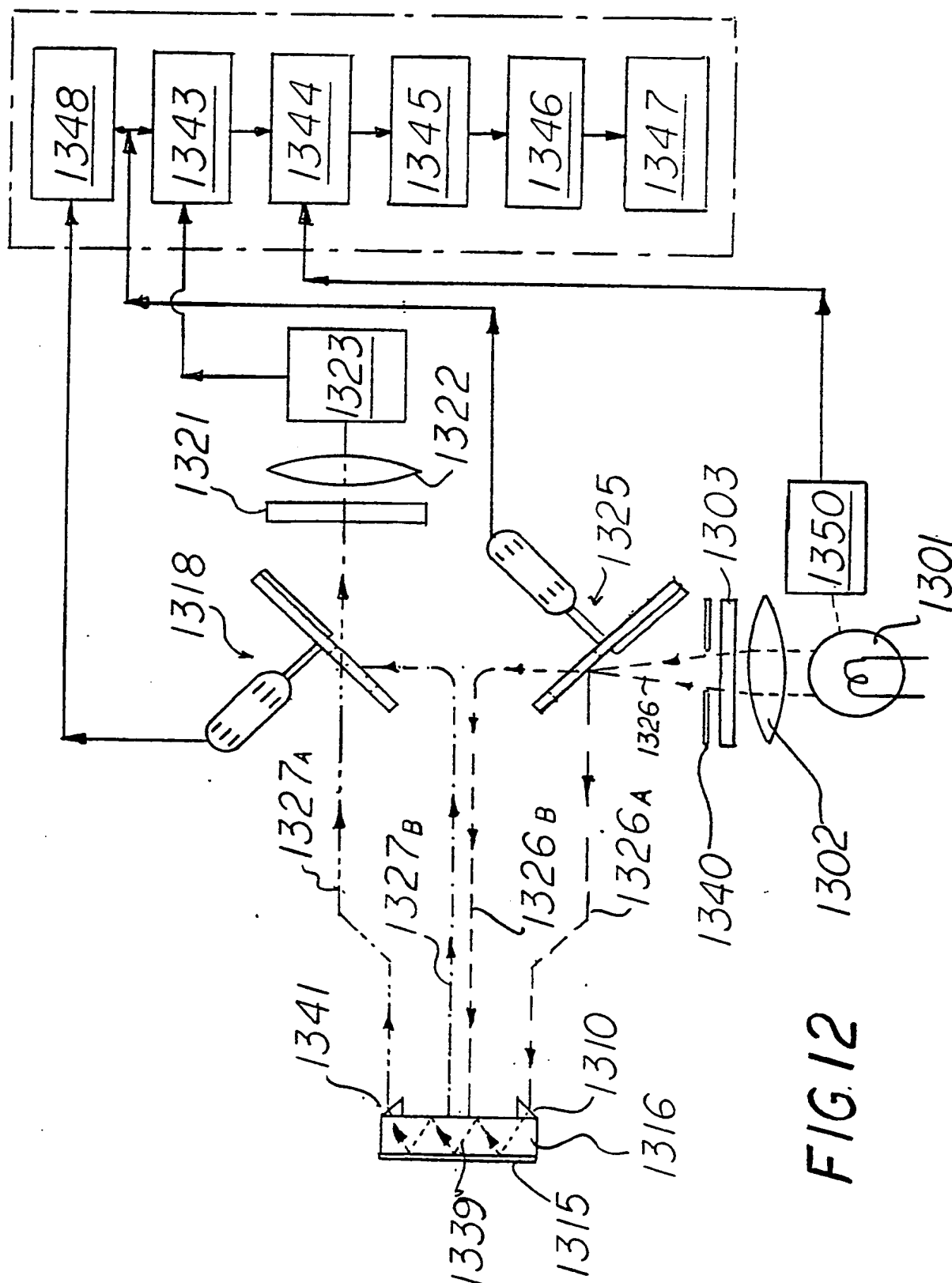


FIG. 12

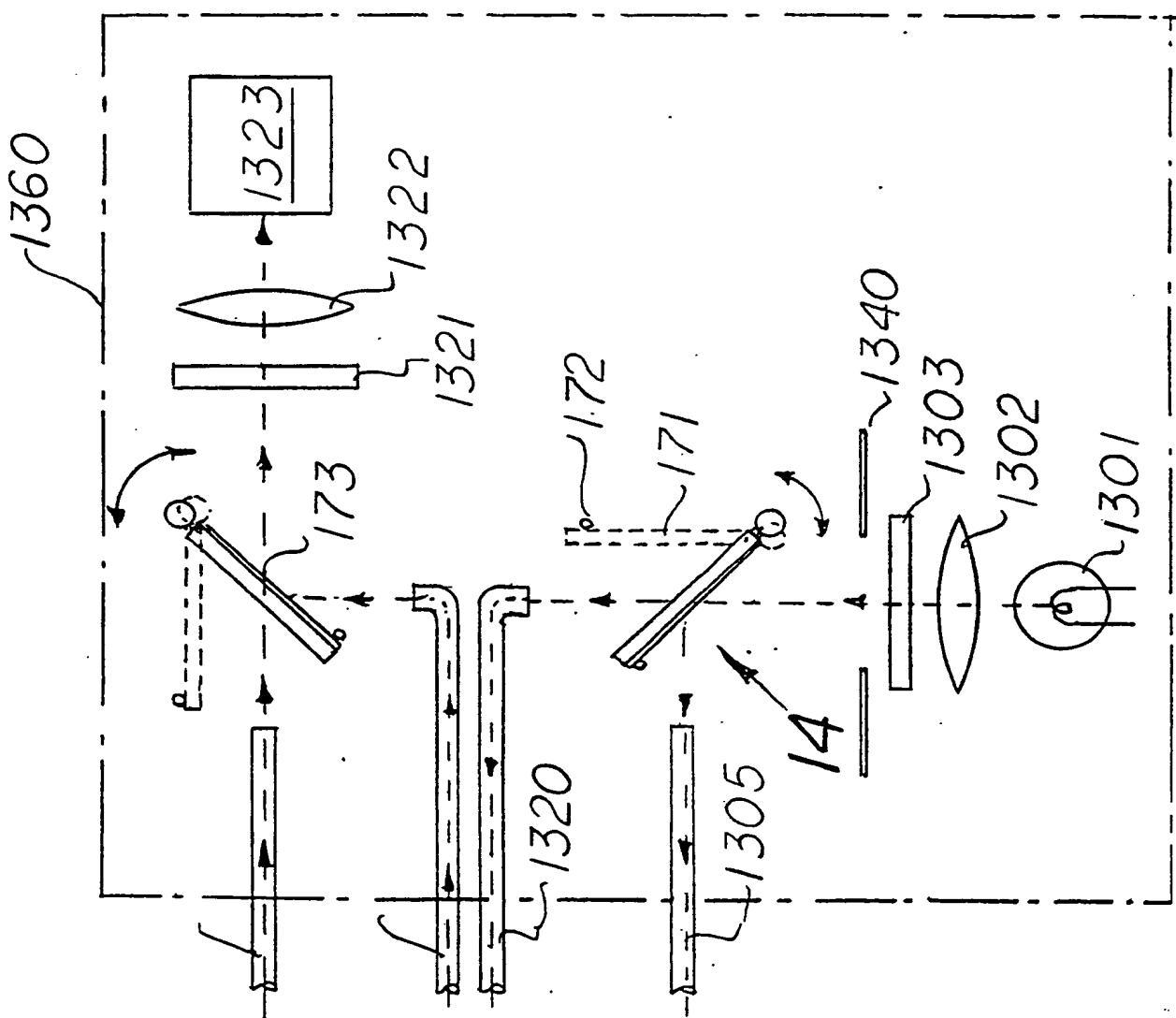
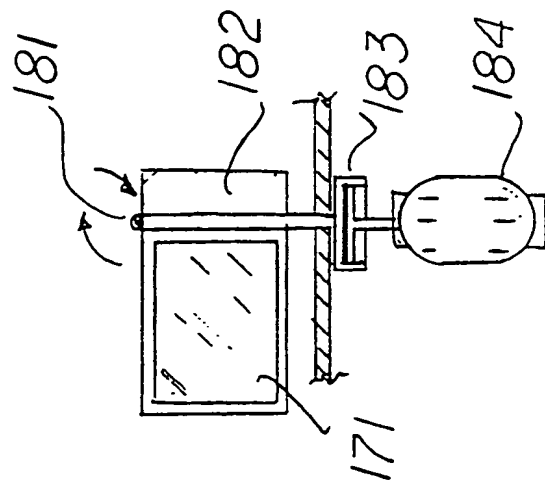
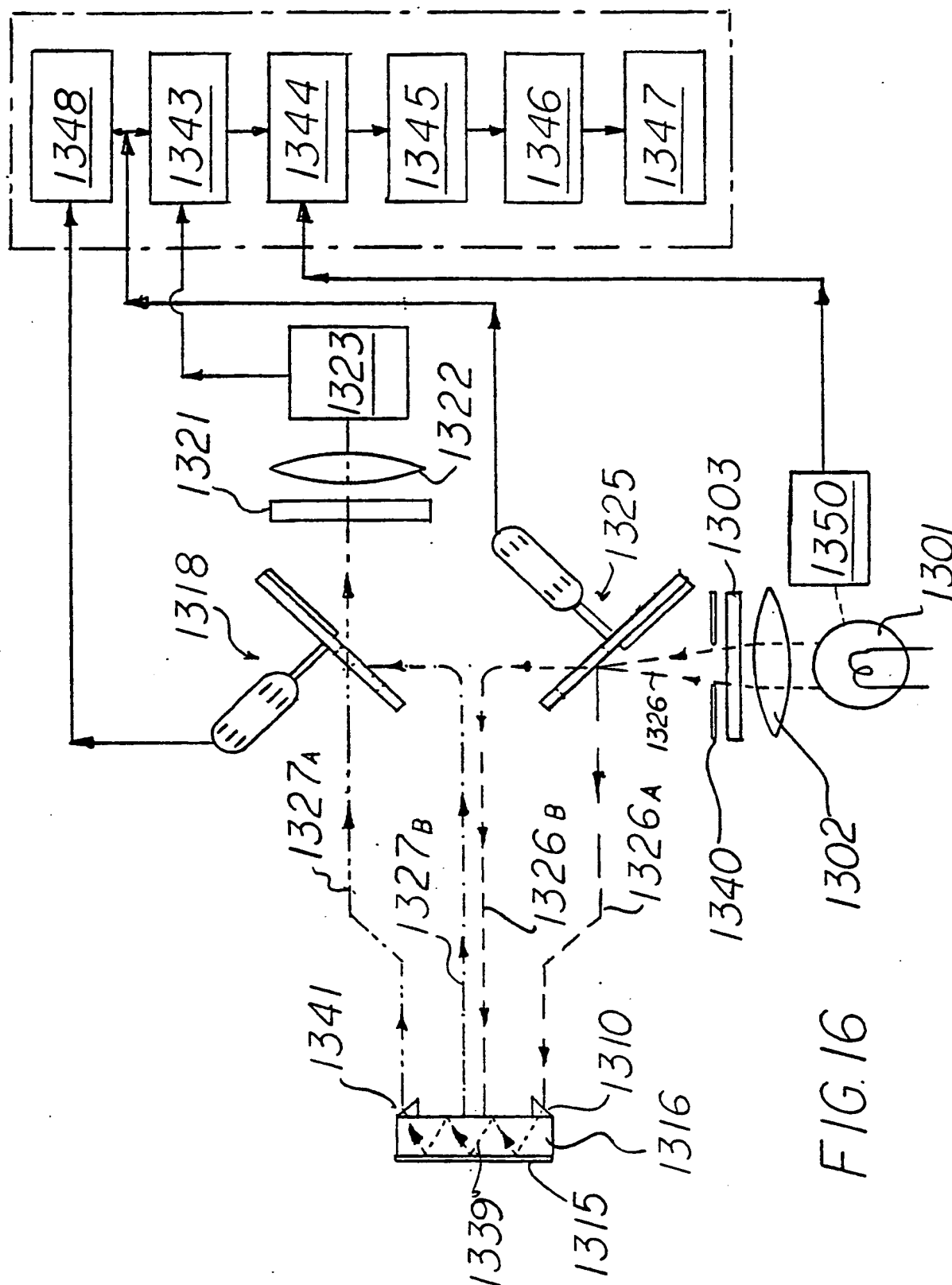


FIG. 14

FIG. 13



SUBSTITUTE SHEET



F/G/16

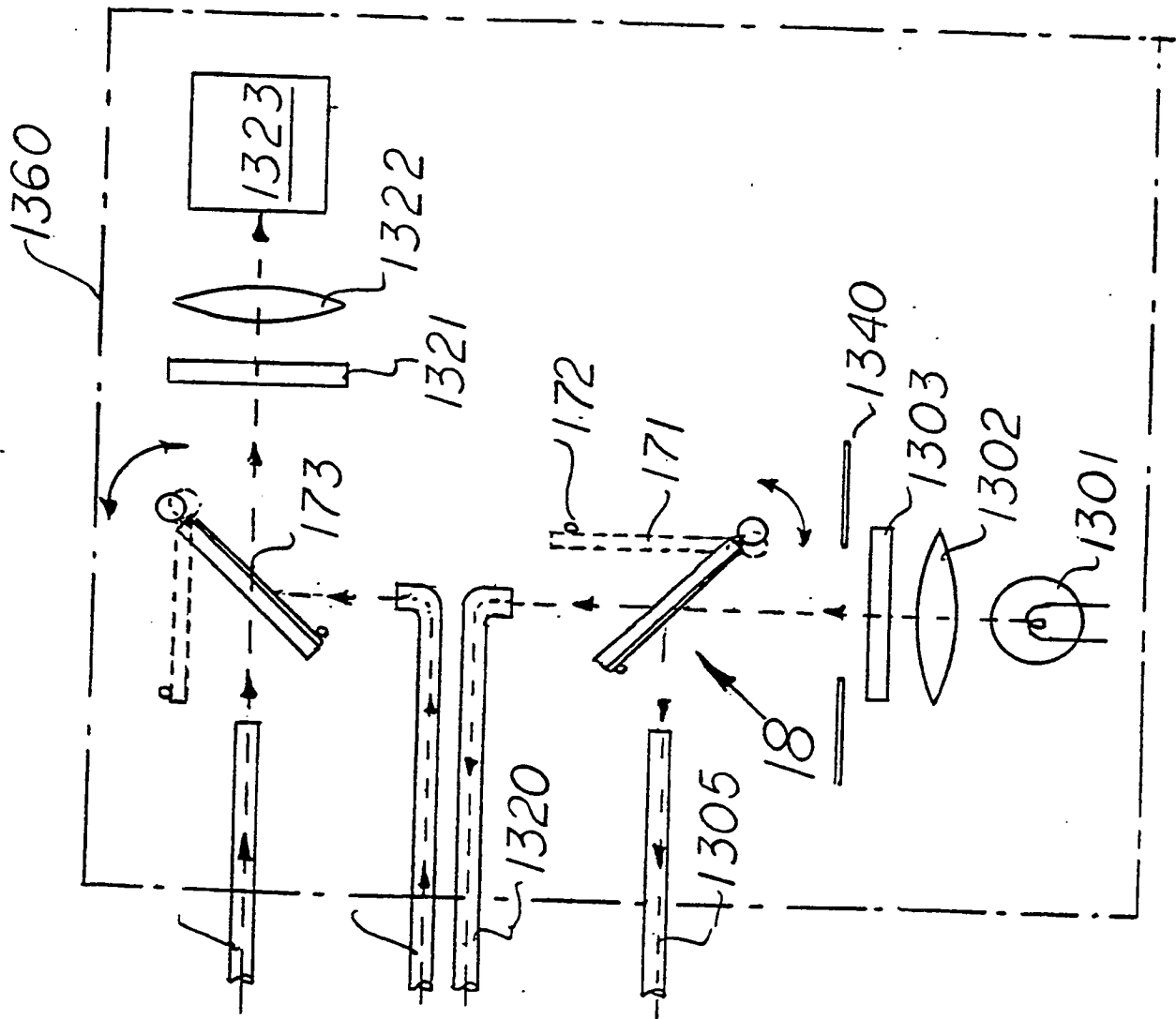
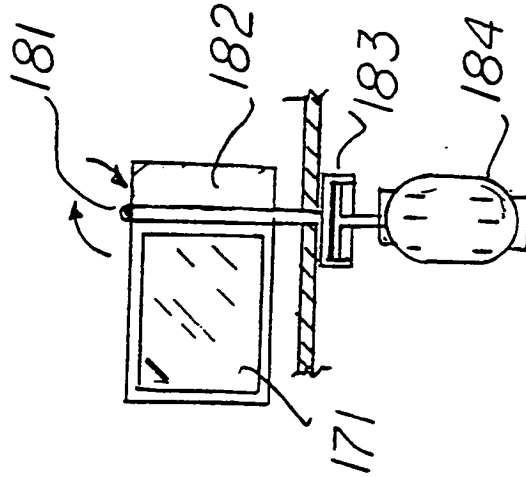


FIG. 18

FIG. 17



INTERNATIONAL SEARCH REPORT

International Application No **PCT/US 87/00088**

I. CLASSIFICATION OF SUBJECT MATTER (If several classification symbols apply, indicate all) ³		
According to International Patent Classification (IPC) or to both National Classification and IPC IPC(4): G01N 21/64 U.S. C1: 250/461.1		
II. FIELDS SEARCHED		
Minimum Documentation Searched ⁴		
Classification System	Classification Symbols	
U.S.	250/343, 373, 461.1, 416.2, 483.1 356/317, 318, 417	
Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched ⁵		
III. DOCUMENTS CONSIDERED TO BE RELEVANT ¹⁴		
Category ⁶	Citation of Document, ¹⁶ with indication, where appropriate, of the relevant passages ¹⁷	Relevant to Claim No. ¹⁸
X	US, A, 3,604,927 (HIRSCHFELD) 14 September 1971	1
A	US, A, 3,902,807 (FLEMING ET AL) 02 September 1975	1-9
A	US, A, 3,939,350 (KRONICK ET AL) 17 February 1976	1-9
A	US, A, 3,975,084 (BLOCK) 17 August 1976	1-9
Y	US, A, 4,447,546 (HIRSCHFELD) 08 May 1984	1-9
Y	US, A, 4,608,344 (Carter et al) 26 August 1986	1-9
<div style="display: flex; justify-content: space-between;"> <div style="width: 45%;"> <p>⁹ Special categories of cited documents: ¹³</p> <p>"A" document defining the general state of the art which is not considered to be of particular relevance</p> <p>"E" earlier document but published on or after the international filing date</p> <p>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</p> <p>"O" document referring to an oral disclosure, use, exhibition or other means</p> <p>"P" document published prior to the international filing date but later than the priority date claimed</p> </div> <div style="width: 45%;"> <p>"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>"X" document of particular relevance: the claimed invention cannot be considered novel or cannot be considered to involve an inventive step</p> <p>"Y" document of particular relevance: the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.</p> <p>"Δ" document member of the same patent family</p> </div> </div>		
IV. CERTIFICATION		
Date of the Actual Completion of the International Search ²	Date of Mailing of this International Search Report ²	
06 April 1987	14 APR 1987.	
International Searching Authority ¹	Signature of Authorized Officer ¹⁰	
ISA/IUS	 Janice A. Howell	

Form PCT/ISA/210 (second sheet) (May 1986)

